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Polymorphisms of the DNA repair gene *MGMT* and risk and progression of head and neck cancer

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Abstract

Methylating agents are involved in carcinogenesis, and the DNA repair protein O⁶-methylguanine-DNA methyltransferase (*MGMT*) removes methyl group from O⁶-methylguanine. Genetic variation in DNA repair genes has been shown to contribute to susceptibility to squamous cell carcinoma of the head and neck (SCCHN). We hypothesize that *MGMT* polymorphisms are associated with risk of SCCHN. In a hospital-based case-control study of 721 patients with SCCHN and 1,234 cancer-free controls frequency-matched by age, sex and ethnicity, we genotyped four *MGMT* polymorphisms, two in exon 3, 16196C>T and 16286C>T and two in the promoter region, 45996G>T and 46346C>A. We found that none of these polymorphisms alone had a significant effect on risk of SCCHN. However, when these four polymorphisms were evaluated together by the number of putative risk genotypes (i.e. 16195CC, 16286CC, 45996GT+TT, and 46346CA+AA), a statistically significantly increased risk of SCCHN was associated with the combined genotypes with three to four risk genotypes, compared with those with zero to two risk genotypes [adjusted odds ratio (OR) = 1.27; 95% confidence interval (CI) = 1.05-1.53]. This increased risk was also more pronounced among young subjects (OR = 1.81; 95% CI = 1.11-2.96), men (OR = 1.24; 95% CI = 1.00-1.55), ever smokers (OR = 1.25; 95% CI = 1.01-1.56), ever drinkers (OR = 1.29; 95% CI = 1.04-1.60), patients with oropharyngeal cancer (OR = 1.45; 95% CI = 1.12-1.87), and oropharyngeal cancer with regional lymph node metastasis (OR = 1.52; 95% CI = 1.16-2.01). In conclusion, our results suggest that any one of *MGMT* variants may not have a substantial effect on SCCHN risk, but a joint effect of several *MGMT* variants may contribute to risk and progression of SCCHN, particularly for oropharyngeal cancer, in non-Hispanic whites.

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Conflict of Interest
None.

Keywords

oral cancer; DNA repair; methylation; genetic susceptibility; molecular epidemiology

1. Introduction

Squamous cell carcinomas of the head and neck (SCCHN), including those of the oral cavity, pharynx, and larynx, are the sixth most frequently occurring cancers and the seventh leading cause of cancer-related deaths worldwide [1]. In the United States, there were estimated to be approximately 48,010 new cases of and 11,260 deaths from SCCHN in 2009 [2]. Although tobacco use and alcohol consumption are the major risk factors for SCCHN [3], only a fraction of exposed individuals develop this disease, suggesting that there exists individual susceptibility to environmental exposure-related carcinogenesis.

The O⁶-methylguanine-DNA methyltransferase (MGMT) is a DNA repair protein that removes a methyl group from the O⁶-position in guanine and transfers it to its own cysteine residue at codon 145 in the protein, inactivating the MGMT protein itself while repairing guanine [4]. Therefore, the MGMT protein plays an important role in removing major premutagenic lesions induced by O⁶-methylating agents, preventing cytotoxicity and defending against both endogenous and exogenous methylating agents [5]. The O⁶-methylguanine, a methylated DNA adduct at the O⁶-position of guanine, may cause a G:C to A:T transition mutation during DNA replication [6], sister chromatid exchanges, and chromosomal aberrations [7]. The detectable levels of the MGMT protein vary in different tissues and types of the cells; in some tumor tissues, MGMT expression appears to be upregulated, compared with the corresponding normal tissue, with increasing tumor grading [8,9], but in others the expression level of the MGMT protein tends to decrease in some tumor tissues [10], particularly in glioma, likely due to promoter methylation [11], although deletion or point mutations and rearrangement of the *MGMT* gene may not necessarily lead to the loss of MGMT activity [12]. Inactivation of the *MGMT* gene by its promoter methylation is one of epigenetic regulation mechanisms in gene expression, a common phenomenon observed in a variety of primary human tumors [10,13], including SCCHN [11,14-19]. It has been reported that the *MGMT* knockout mice had a higher incidence of nitrosamine-induced tumorigenesis [20]. Recently, loss of MGMT expression was found to be common in oral leukoplakia and early oral cancer [21] and SCCHN [22] and was associated with their progression [23]. Taken together, these data suggest that altered *MGMT* expression may modulate susceptibility to SCCHN.

The *MGMT* gene is mapped on chromosome 10q26 and spans at least 15 kb [24,25]. To date, a total of 1964 polymorphisms in human *MGMT* gene have been described (<http://www.ncbi.nlm.nih.gov/projects/SNP>), but only two common, potentially functional polymorphisms in exon 3 (rs1803965C>T Leu53Leu and rs12917C>T Leu84Phe) (GenBank accession no: AL157832) [26-28] have been investigated for their association with cancer risk [29,30], because they are commonly detected in all ethnic groups (Table 1). However, few studies have investigated two new *MGMT* promoter polymorphisms: rs1711646C>A (formerly named 135G>T) and rs1625649G>T (formerly named 485C>A) (GenBank accession no: AL355531) (Table 1).

Most published studies have reported that both Leu53Leu and Leu84Phe polymorphisms are not associated with cancer risk [27,31-34]. In a US study of lung cancer, we did not observe any main effect of the selected four polymorphisms (135G>T, 485C>A, Leu84Phe and Ile143Val, none of which was located on CpG methylation islands) on the risk [35]. In a subsequent Chinese study of lung cancer with 39 *MGMT* variants as well as a subset of 10 haplotype-tagging SNPs (htSNP) and three pre- and interblock SNPs to capture variation across

MGMT, we did not observe an association between cancer risk and any of these variants [36]. However, one study reported that both Leu84Phe and Ile143Val was associated with a decreased risk of SCCHN in a pooled analysis of US populations [37], but in other studies these two variants were not associated with oral cancer nor with secondary cancer. Furthermore, we found a significant increased bladder cancer risk associated with the combined genotypes of Leu53Leu and Leu84Phe polymorphisms [38].

To date, no reported study has investigated the association between the *MGMT* promoter 135G>T and 485C>A polymorphisms and risk of SCCHN. In the present study, we hypothesized that these two *MGMT* promoter polymorphisms contribute to risk of SCCHN, and we tested this hypothesis in our ongoing hospital-based case-control study of SCCHN. Because we observed a combined effect of Leu53Leu and Leu84Phe on risk of bladder cancer in a Chinese population [38], suffering from tobacco-induced cancer, we wished to replicate this finding for SCCHN in a US population.

2. Materials and methods

2.1. Study subjects

The recruitment of study subjects has been previously described [39]. Briefly, the study population included 721 patients with newly diagnosed SCCHN and 1 234 cancer-free control subjects recruited between May 1995 and September 2003. Approximately 95% of the eligible patients contacted chose to participate in this study. Only non-Hispanic white patients were included in this analysis because genotype frequencies can vary between ethnic groups and few patients of ethnic minority groups were recruited. Among the 721 SCCHN patients included in the analysis, 222 (30.8%) had cancers of the oral cavity, 326 (45.2%) had cancers of oropharynx, and 173 (24.09%) had cancers of larynx (including 37 of the hypopharynx). Patients with second SCCHN primary tumors, primary tumors of the nasopharynx or sinonasal tract, primary tumors outside the upper aerodigestive tract, cervical metastases of unknown primary origin, or histopathologic diagnoses other than SCC were excluded. The regional lymph node (N) involvement was defined as follows [40]: N₀, no regional node metastasis; N₁, metastasis in a single ipsilateral lymph node, 3 cm or less in greatest dimension; N₂, metastasis in a single ipsilateral lymph node, more than 3 cm but not more than 6 cm in greatest dimension; or in multiple ipsilateral lymph nodes, none more than 6 cm in greatest dimension; or in bilateral or contralateral lymph nodes, none more than 6 cm in greatest dimension; N₃, metastasis in a lymph node more than 6 cm in greatest dimension.

Cancer-free control subjects were recruited from the biologically unrelated individuals who were not seeking health care but accompanying the case patients to visit the clinics. We first surveyed potential control subjects at the clinics using a short questionnaire to determine their willingness to participate in research studies and to obtain demographic and risk factor information. We frequency matched the controls to the cases by age (± 5 years) and sex. Among the willing respondents we contacted for recruitment, the response rate was greater than 80%. We interviewed each eligible subject to obtain data on tobacco smoke and alcohol use. After signing informed consent forms, each subject donated 30 ml of blood, of which 1 ml used for genomic DNA extraction with a DNA blood Mini Kit (Qiagen Inc., Valencia, CA) according to the manufacturer's instructions. The research protocol was approved by the M. D. Anderson institutional review board.

2.2. Genotyping

We determined the four *MGMT* polymorphisms by using the polymerase chain reaction-restriction fragment length polymorphism method as previously described [38], which showed that in these *MGMT* genotyping assays the genotyping gel pictures were very clear and easy

to distinguish the genotypes in a double-blinded reading by two different lab personnel (the first and second authors). The *Hpy188I*, *EarI*, *ApeKI*, and *BanI* restriction enzymes (New England Biolabs, Beverly, MA) were used to distinguish the 16196C>T, 16286C>T, 45996G>T, and 46346C>A, respectively, which resulted in 121-bp and 58-bp fragments in the presence of 16196C allele; 68-bp, 30-bp, and 4-bp fragments in the presence of 16286C allele; 168-bp, 44-bp, and 32-bp in the case of 45996G allele; and 139-bp and 73-bp in the case of 46346A allele. More than 10% of the samples were randomly selected for confirmation, and the results were 100% concordant. The genotypes of 45996G>T, and 46346C>A were confirmed by direct sequencing (Figure 1)

2.3. Statistical analysis

We used Chi-square test to evaluate the differences in the frequency distributions of selected demographic variables, smoking status, alcohol use, and each allele and genotype of the four *MGMT* polymorphisms between the cases and controls. Unconditional univariate and multivariate logistic regression analyses were performed to obtain the crude and adjusted odds ratios (ORs) and 95% confidence intervals (CIs). The multivariate adjustment included age, sex, smoking status, and alcohol use. Considering the potential interaction of the *MGMT* polymorphisms on SCCHN risk, the associations between the combined genotypes of the four polymorphisms and SCCHN risk were evaluated. The 2LD software was used to calculate the D' value among the four polymorphisms [41,42]. The combined genotype data were further stratified by subgroups of age, sex, smoking status, alcohol use, and the primary tumor sites, e.g., oral cavity, the oropharynx, and larynx including hypopharynx. Two-sided tests of statistical significance were conducted by using the SAS software (version 8.0; SAS Institute, Inc., Cary, NC).

3. Results

The frequency distributions of selected characteristics of the cases and controls are presented in Table 2. The cases and controls appeared to be well matched on age and sex: the mean age was 57.0 years for cases (± 11.9 years; range, 18-90 years) and 57.1 years for controls (± 11.6 years; range, 20-87 years) ($P = 0.287$), and 74.9% and 25.1% of the cases and 74.1% and 25.9% of the controls were men and women, respectively ($P = 0.686$). However, there were more current smokers (34.8%) and current drinkers (51.2%) among the cases than among the controls (25.4% and 43.7%, respectively), and these differences were statistically significant ($P < 0.001$). Therefore, these variables were further adjusted for in the multivariate logistic regression analysis for any residual confounding effect.

3.1. Genotype distributions of the *MGMT* polymorphisms between the cases and controls

Genotype distributions and allele frequencies of the four *MGMT* polymorphisms in case patients and control subjects and their associations with SCCHN risk are summarized in Table 3. We found no significant difference in the genotype distributions between the cases and controls ($P = 0.671$ for 16195C>T, $P = 0.395$ for 16286C>T, $P = 0.365$ for 45996G>T, and $P = 0.183$ for 46346C>A). The genotype frequencies of these four polymorphisms among the controls were all in agreement with the Hardy-Weinberg equilibrium (chi-square test: $P = 0.590$ for 16195C>T, $P = 0.377$ for 16286C>T, $P = 0.606$ for 45996G>T, and $P = 0.057$ for 46346C>A).

As shown in Table 3, the frequencies of the 45996T and 46346A alleles (0.178 and 0.359, respectively) among the cases were slightly higher than those among the controls (0.172 and 0.341, respectively), and in contrast, the frequencies of the 16195T and 16286T alleles (0.108 and 0.114, respectively) among the cases were slightly lower than those among the controls (0.118 and 0.129, respectively), suggesting the 45996T, 46346A 16195C, and 16286C alleles

may be the putative risk alleles to be considered in further combined analysis. Although none of the variant genotypes was associated with significantly altered risk, both 45996T and 46346A alleles tended to be associated with non-significantly increased SCCHN risk (OR = 1.09 and 95% CI = 0.89-1.32 for 45996GT+TT and OR = 1.17 and 95% CI = 0.99-1.47 for 46346CA+AA), whereas both 16195T and 16286T alleles tended to be associated with non-significantly reduced SCCHN risk (OR = 0.89 and 95% CI = 0.71-1.12 for 16195TT+TT and OR = 0.87 and 95% CI = 0.70-1.08 for 16286CT+TT).

3.2. Distribution of the MGMT combined genotypes between the cases and controls

The LD analysis revealed that the alleles of two polymorphisms in exon 3 (i.e. 16195C>T, rs1803965 and 16286C>T, rs12917) were in LD ($D' = 0.93$ and $R^2 = 0.78$), but the two polymorphisms in the promoter region (i.e. 45996G>T, rs1711646 and 46346C>A, rs1625649) were not (Figure 2A and 2B), among the controls. Because rs12917 (Leu84Phe) is a non-synonymous SNP, we did a mini pool analysis of existing literature (Figure 3) and found that the result of our overall risk estimate of the variant genotypes (84Phe, CT+TT) for SCCHN (0.87, 0.70-1.08), the largest US study (the present study, Zhang 2009), was similar to that of another US study [37] (0.78, 0.61-1.00) and a Thailand study [43] (1.00, 0.55-1.83) but somewhat different from that of an European study [44] (1.60, 1.24-2.05). Overall, this single variant was not associated with risk of SCCHN (1.02, 0.71-1.46) based on 1757 cases and 2901 controls in our pooled analysis of existing literature.

Considering possible combined effects from different variants or genotypes and potential interactions of *MGMT* polymorphisms on risk of SCCHN, we combined these four *MGMT* polymorphisms by the number of the putative risk genotypes (i.e. 16195CC, 16286CC, 45996GT+TT, and 46346CA+AA). As shown in Table 4, there were more individuals with three risk genotypes and fewer individuals with two risk genotypes among the cases (49.0% and 22.5%, respectively) than among the controls (43.9% and 27.3%, respectively), and these differences were statistically significant ($P = 0.014$). When we dichotomized the combined genotypes into two groups by the number of risk genotypes (i.e. 0-2 risk genotypes vs. 3-4 risk genotypes), we found that the difference in the distribution of the combined genotypes between the cases and controls was statistically significant ($P = 0.012$).

3.3. Association and stratification analysis between the MGMT combined genotypes and risk of SCCHN

As shown in Table 5, we found that the combined genotype with 3-4 risk genotypes was associated with a statistically significantly increased risk of SCCHN compared with those with 0-2 risk genotypes (OR = 1.27; 95% CI = 1.05-1.53). In the stratification analysis, we found that the risk of SCCHN associated with 3-4 risk genotypes was decreased in a dose-response manner as age increased; that is, the youngest subjects (≤ 45 years) with 3-4 risk genotypes had the highest risk compared with those with 0-2 risk genotypes (OR = 1.81; 95% CI = 1.11-2.96 for aged ≤ 45 years, OR = 1.21; 95% CI = 0.91-1.61 for aged 46-60 years, and OR = 1.16; 95% CI = 0.86-1.58 for aged > 60 years). This increased risk was also more pronounced among men (OR = 1.24; 95% CI = 1.00-1.55), ever smokers (OR = 1.25; 95% CI = 1.01-1.56), ever drinkers (OR = 1.29; 95% CI = 1.04-1.60), and patients with oropharyngeal cancer (OR = 1.45; 95% CI = 1.12-1.87) than other subgroups in the same stratum (Table 5). However, no statistical evidence was found for any interactions between the combined genotypes and these variables (i.e. age, sex, smoking status, and alcohol use; data not shown).

3.4. Association between the MGMT combined genotypes and progression of SCCHN

Because the risk associated with the combined genotype with 3-4 risk genotypes was more pronounced in oropharyngeal cancer, we then evaluated whether these polymorphisms had an effect on tumor progression. As shown in Table 6, when we used the combined genotype with

0-2 risk genotypes as the reference, we found that the combined genotype with 3-4 risk genotypes was associated with a statistically significantly increased risk of oropharyngeal cancer with regional lymph nodes metastasis (OR = 1.52; 95% CI = 1.16-2.01) but not oropharyngeal cancer without regional lymph nodes metastasis (OR = 1.10; 95% CI = 0.61-1.97). In addition, we found also that the combined genotype with 3-4 risk genotypes was associated with a statistically significantly increased risk of early-stage (T₁₋₂) oropharyngeal cancer (OR = 1.77; 95% CI = 1.28-2.44) but not late-stage (T₃₋₄) oropharyngeal cancer (OR = 1.09; 95% CI = 0.76-1.58). These were not observed for cancers of the oral cavity and larynx/hypopharynx (data not shown).

Finally, because multiple tests had been performed, we calculated the false positive reporting probability. As shown in Table 7, the positive findings for all subjects, ever smoker, and ever drinking had a power greater than 80%, and they were unlikely chance findings.

4. Discussion

Our finding of no main effects of each *MGMT* variant on risk of SCCHN are consistent with previously published studies [27,31-34], particularly for a large study of the upper aerodigestive tract (UADT) [44], in which no main effects of Leu53Leu and Leu84Phe were found on risk of SCCHN (n=438, including 117 oral cavity, 85 pharynx and 236 larynx), compared with 529 controls. However, our finding of a non-significant protective effect from 16195T (53Leu) and 16286T (84Phe) alleles is also consistent with those reported in the pooled analysis of SCCHN of 555 cases (430 whites) and 792 controls (695 whites) [37]. None of these studies have investigated the effects of the promoter SNPs or combined variant genotypes on SCCHN risk as in the present study that had the largest numbers of cases and controls (Figure 2). Our results suggest that any one of the *MGMT* variants may not have a substantial effect on SCCHN risk, but a joint effect of several *MGMT* variants may contribute to risk and progression of SCCHN, particularly for oropharyngeal cancer, in non-Hispanic whites. Given the role the *MGMT* gene may play in carcinogenesis, it is plausible that the *MGMT* polymorphisms may modulate risk of SCCHN.

MGMT plays an important role in maintaining genomic integrity, and *MGMT* is a suicide repair enzyme that is responsible for removing DNA damage induced by methylating agents [4], and variation in its enzyme activity has been observed in SCCHN cell lines [45]. In the present study, we found that those who carried the combined genotypes (i.e., 3 or 4 of the 16195CC, 16286CC, 45996GT+TT, and 46346CA+AA genotypes) with a greater number of risk genotypes appeared to be at increased risk of SCCHN, although these polymorphisms individually did not have a significant main effect. Specifically, the individuals with three to four risk genotypes had a higher risk of SCCHN than those with zero to two risk genotypes, and this increased risk was more pronounced among men, ever smokers, ever drinkers, and patients with oropharyngeal cancer with regional lymph nodes metastasis.

Although how the two common *MGMT* polymorphisms in exon 3 affect the enzyme activity remains to be investigated, some studies suggested that these two polymorphisms might affect the protein function. It is reported that melanoma patients with the *MGMT* 16195T/16286T variant alleles in the promoter had a high level of *MGMT* expression and that these two polymorphisms may affect the methylation status of the *MGMT* gene and thus may have an effect on *MGMT* protein expression and activity [32]. Recently, one study reported that the level of *MGMT* expression was associated with the sensitivity of human oral cancer cell lines to the alkylating agents, such as the anticancer drug, cis-diaminedichloroplatinum, suggesting that abnormal *MGMT* expression may affect the DNA repair capacity and sensitivity in response to chemotherapy of SCCHN [45]. Our finding of a positive association of select *MGMT* polymorphisms with risk of SCCHN further supports the notion that some of these

MGMT polymorphisms, or other untyped ones they may be in linkage with, may have an effect on the *MGMT* activity. A possible explanation for the increased risk of SCCHN is that the *MGMT* 84Phe mutant may have an effect on the *MGMT* Zn²⁺ binding (25) and thus on its repair function and response to exposure to smoking (26). The other is that the *MGMT*-dependent repair of the methylated DNA damage is weakened by the *MGMT* risk alleles or those they represent as a result of affected *MGMT* protein expression and activity, particularly for the *MGMT* polymorphisms in the promoter region. However, this hypothesis needs to be confirmed by future functional studies.

The findings of no single polymorphism having a significant main effect on risk of SCCHN are consistent with our previous study [38] and other published data [27,31,32,46]. Several studies have reported that the variant genotype distributions of the 16286C>T between the cases and controls were not significantly different [31,33,34]. In the association studies, one study reported that the 16286C>T polymorphism was not associated with increased risk of lung cancer [46], but its small sample size (96 cases and 96 controls) did not have enough statistical power to reveal any significant ORs. However, a recent study demonstrated that the variant genotypes of the 16286C>T was associated with a significantly decreased risk of the head and neck cancer in a population-based case-controls study [37]. The discrepancies in these reported studies could be due to differences in study design and inclusion of different ethnic groups (Table 1). For example, our results indicate that the genotype distributions of the *MGMT* polymorphisms vary with ethnicity. The frequencies of the CC and CT genotypes of the 16286C>T among our non-Hispanic whites were 75.6% and 23.0%, respectively, compared with 84.8% and 13.7%, respectively, of our previous study of 204 southern Chinese controls [38], 89.0% and 11.9%, respectively, of 100 southern Chinese population in the studies by Liu *et al.* [33,34], and 83.6% and 16.2%, respectively, of 225 Japanese population in the study by Otsuka *et al.* [26]. However, these ethnic variations in genotype distributions and their influence on the risk of cancer need to be further investigated.

In this study, we found that the SCCHN risk associated with the combined genotype with 3-4 risk genotypes was more pronounced in oropharyngeal cancer, particularly for those with regional lymph node metastasis, suggesting that the putative risk genotypes may be associated with the progression of oropharyngeal cancer or perhaps with human papillomavirus-associated oropharyngeal cancer (a disease almost always presenting with nodal metastases). However, this finding from subgroup analysis could be due to chance. Larger studies are needed to verify this finding. We found a higher risk in ever smokers and ever drinkers, suggesting the risk-genotype carriers were at greater risk if they had continuous exposure. However, there was no evidence of a gene-environment interaction, that is, tobacco smoke and alcohol use did not modify the risk associated with the *MGMT* combined genotypes.

The strengths of our study include its relatively large sample size, use of combined risk genotypes, and inclusion of the known polymorphisms within the exon and promoter region of *MGMT* gene. Because our study was hospital-based, limitations inherent in the case-control study design could introduce selection bias compared with population-based studies. However, the genotype distributions in our study were similar to distributions reported in other studies. For instance, the frequencies of the CC, CT, and TT genotypes of the 16286C>T among our 1 234 non-Hispanic white control subjects were 75.6%, 23.0%, and 1.4%, respectively, compared with 70.4%, 26.9%, and 2.7%, respectively, of 665 white control subjects in the study by Huang *et al.* [37]. Similarly, the genotype frequency of the 16195CT+TT among our control subjects was 22.0% compared with 21.0% of 76 healthy Swedish population [32]. So far, there are no reported frequencies of the 45996G>T and 46346C>A genotypes among Caucasian populations to compare with. Because the genotype frequencies of the 16195C>T and 16286C>T polymorphisms estimated from the hospital-based control subjects in our study were very close to those of the population-based control subjects, any selection bias in genotype

distribution is unlikely to be substantial. However, the positive findings in stratification analysis may need further validation in larger studies.

5. Conclusion

We did not find any significantly increased risk of SCCHN associated with any of the four *MGMT* polymorphisms (i.e., 16196C>T, 16286C>T, 45996G>T, and 46346C>A), when they were analyzed individually. This is consistent with published literature in which the SNPs of *MGMT* either exhibited modest effects on the risk of cancer, or reportedly functional *MGMT* SNPs did not have a major effect on protein function [29]. However, given only a modest effect of each SNP individually, evaluating their combined effects may help us better understand any role of *MGMT* SNPs may have in cancer etiology. Indeed, we found that the combined genotypes of these four polymorphisms (i.e., 16195CC, 16286CC, 45996GT+TT, and 46346CA+AA most risky genotypes, compared with the 16195TT+CT, 16286TT+CT, 45996GG, and 46346CC less risky genotypes) were associated with a statistically significantly increased risk of SCCHN, and this increased risk was more pronounced among the young, men, ever smokers, ever drinkers, and patients with oropharyngeal cancer. Although the significance of these findings from subgroup analyses may be limited, the results do suggest that the risk genotypes of the 16196C>T, 16286C>T, 45996G>T, and 46346C>A polymorphisms may jointly contribute to risk and perhaps progression of SCCHN, particularly for oropharyngeal cancer. Larger studies that include more detailed data on environmental exposure (such as human papillomavirus) and more oropharyngeal cancers are required to verify these findings.

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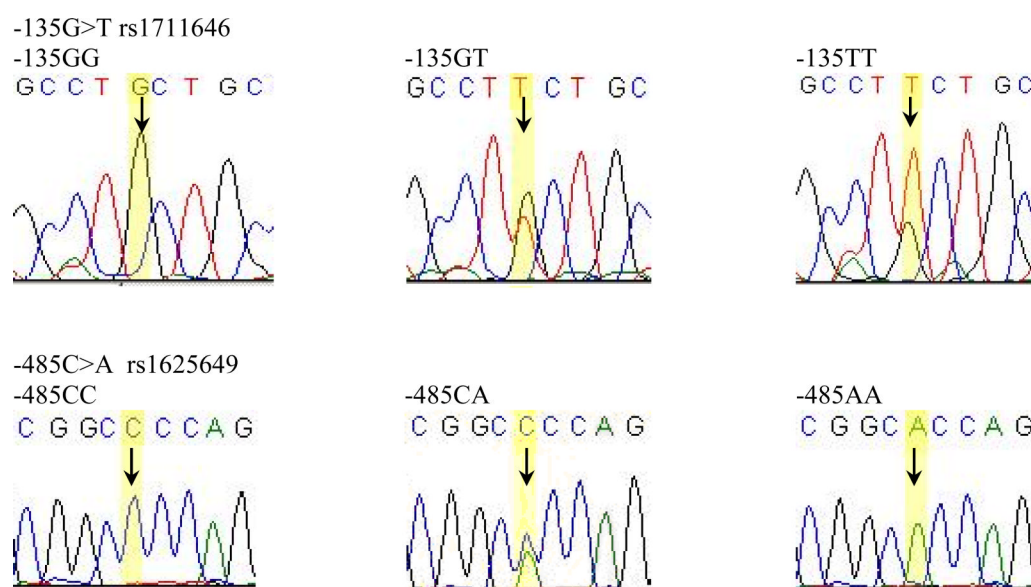


Fig 1.
Direct sequencing results for the *MGMT* -135G>T (GG, GT and TT) and -485C>A (CC, CA and AA)

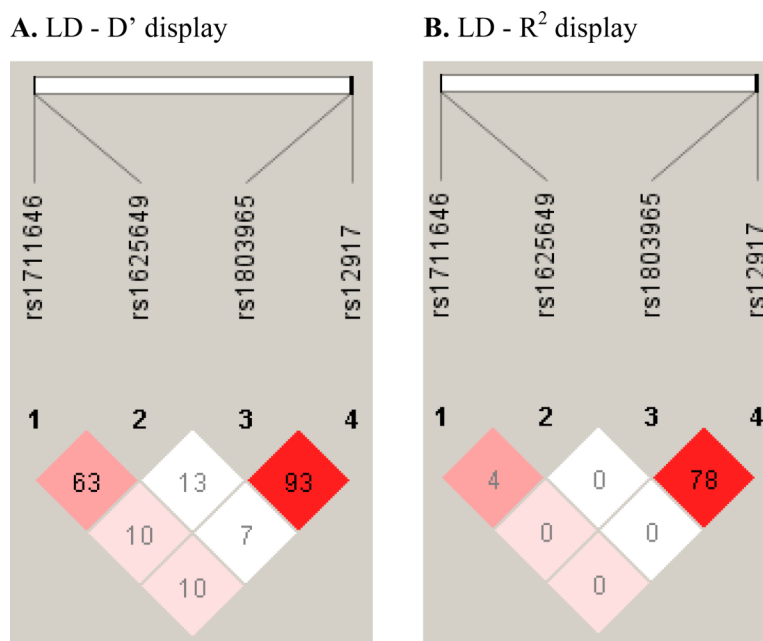


Fig. 2. Linkage disequilibrium (LD) display for the four *MGMT* variants. **A**, the D' display and **B**, the R² display, both showed that rs1803965 (Leu53Leu53) and rs12917 (Leu84Phe) are in LD with a D' = 0.93 and R² = 0.78

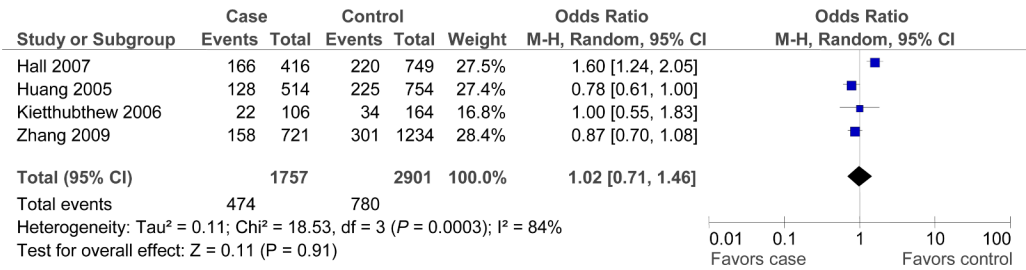


Fig. 3. Meta-analysis of associations between the functional *MGMT* Leu84Phe (rs12917) variant and risk of SCCHN. The result of our overall risk estimate for SCCHN, the largest US study (Zhang 2009), was similar to that of another US study (Huang 2005) and a Thailand study (Kietthubthew 2006) but somewhat different from that of an European study (Hall 2007). Overall, this single variant was not associated with risk of SCCHN.

Table 1

Potentially functional SNPs of *MGMT* studied for cancer

SNPs in MGMT reported in literature ^[29]	SNPs in MGMT reported in dbSNP ^d	Base change ^a	rs number	Location on chromosome 10 (nt position) ^a	MAF in the HapMap for YRI/JPT/CHB/CEU ^b
135G>T ^[35]	5' near gene	A>G	rs10764881	promoter	A: 0.025/0.0/0.0/0.0
485C>T ^[35]	5' near gene	C>A	rs1711646 ^c	promoter (45996) ^d	no information
Glu30Lys	5' near gene	G>T	rs1625649 ^c	promoter (46346) ^d	no information
Leu53Leu	Glu61Lys	G>A	rs2020893	exon 4	A: 0.009/0.0/0.0/0.0
Trp65Cys	Leu84Leu	C>T	rs1803965 ^c	exon 5 (16195) ^e	T: 0.181/0.159/0.133/0.085
Leu84Phe	Trp96Cys	G>C	rs2282164	exon 5	C: 0.0/0.012/0.0/0.0
Arg128Gln	Leu115Phe	C>T	rs12917 ^c	exon 5 (16286) ^e	T: 0.165/0.176/0.114/0.105
Ile143Val	Arg159Gln	G>A	rs3750824	exon 6	A: 0.005/0.018/0.006/0.014
Gly160Arg	Ile174Val	A>G	rs2308321	exon 7	G: 0.0/0.0/0.006/0.159
Glu166Asp	Gly191Arg	G>A	rs2308318	exon 7	no information
Lys178Arg	Glu197Asp	A>T	rs2308320	exon 7	T: 0.008/0.0/0.033/0.0
	Lys209Arg	A>G	rs2308327	exon 7	G: 0.0/0.0/0.006/0.159

^a <http://www.ncbi.nlm.nih.gov/projects/SNP>.^b http://hapmap.ncbi.nlm.nih.gov/cgi-perl/gbrowse/hapmap27_B36; MAF: minor allele frequency; YRI: Yoruba in Ibadan, Nigeria; JPT: Japanese in Tokyo, Japan; CHB: Han Chinese in Beijing, China; CEU: CEPH (Utah residents with ancestry from northern and western Europe).^c Tested in this study.^d Based on AL355531.^e Based on AL157832.

Table 2
Frequency distributions of selected variables in SCCHN cases and cancer-free controls

Variables	Cases (<i>n</i> = 721)		Controls (<i>n</i> = 1,234)		<i>P</i> ^a
	<i>n</i>	%	<i>n</i>	%	
Age (years)					0.287
≤ 45	105	14.6	195	15.8	
46-55	227	31.5	345	27.9	
56-65	220	30.5	371	30.1	
> 65	169	23.4	323	26.2	
Sex					0.686
Female	181	25.1	320	25.9	
Male	540	74.9	914	74.1	
Smoking status					< 0.001
Never	188	26.1	371	30.1	
Former	282	39.1	549	44.5	
Current	251	34.8	314	25.4	
Alcohol use					< 0.001
Never	158	21.9	374	30.3	
Former	194	26.9	321	26.0	
Current	369	51.2	539	43.7	

^aTwo-sided chi-square test.

Genotype and allele frequencies of the *MGMT* polymorphisms among cases and controls and their associations with risk of SCCHN

Table 3

Variant genotypes	Cases (n = 721)		Controls (n = 1,234) ^a		<i>p</i> ^b	Crude OR (95% CI)	Adjusted OR (95% CI) ^c
	n	%	n	%			
rs1711646, 135G>T, 45996G>T							
GG	482	66.9	849	68.8	0.365	1.00	1.00
GT	221	30.6	346	28.0		1.13 (0.92-1.38)	1.12 (0.92-1.38)
TT	18	2.5	39	3.2		0.81 (0.46-1.44)	0.77 (0.69-1.36)
GT+TT^d	239	33.1	385	31.2	0.372	1.09 (0.90-1.33)	1.09 (0.89-1.32)
T allele	0.178		0.172		0.665		
rs1625649, 485C>A, 46346C>A							
CC	294	40.8	550	44.6	0.183	1.00	1.00
CA	337	46.7	525	42.5		1.20 (0.99-1.46)	1.21 (0.99-1.47)
AA	90	12.5	159	12.9		1.06 (0.79-1.42)	1.06 (0.78-1.42)
CA+AA^d	427	59.2	684	55.4	0.102	1.17 (0.97-1.41)	1.17 (0.97-1.41)
A allele	0.359		0.341		0.269		
rs1803965, Leu53Leu, 16195C>T							
CC ^d	575	79.7	963	78.0	0.671	1.00	1.00
CT	136	18.9	252	20.4		0.90 (0.72-1.14)	0.89 (0.71-1.13)
TT	10	1.4	19	1.6		0.88 (0.41-1.91)	0.87 (0.40-1.88)
CT+TT	146	20.3	271	22.0	0.373	0.90 (0.72-1.13)	0.89 (0.71-1.12)
T allele	0.108		0.118		0.370		
rs12917, Leu84Phe, 16286C>T							
CC ^d	563	78.1	933	75.6	0.395	1.00	1.00
CT	151	20.9	284	23.0		0.88 (0.70-1.10)	0.88 (0.71-1.10)
TT	7	1.0	17	1.4		0.68 (0.28-1.65)	0.64 (0.26-1.56)
CT+TT	158	21.9	301	24.4	0.212	0.87 (0.70-1.08)	0.87 (0.70-1.08)
T allele	0.114		0.129		0.185		

^aThe observed genotype frequency among the control subjects was in agreement with the Hardy-Weinberg equilibrium (chi-square = 0.29, *P* = 0.590 for 16195C>T, chi-square = 0.78, *P* = 0.377 for 16286C>T, chi-square = 0.27, *P* = 0.606 for 45996G>T, and chi-square = 3.62, *P* = 0.057 for 46346C>A).

^b Two-sided chi-square test for either genotype distribution or allele frequency.
^c Obtained from logistic regression model with adjustment for age, sex, smoking status, and alcohol use.
^d Assumed risk genotypes.

Table 4
Distributions of the *MGMT* combined genotypes between the SCCHN cases and controls

No. of risk genotypes ^a	Cases (<i>n</i> = 721)		Controls (<i>n</i> = 1,234)		<i>p</i> ^b
	<i>n</i>	%	<i>n</i>	%	
Ordinal					0.014
0	22	3.0	65	5.3	
1	105	14.5	165	13.4	
2	162	22.5	337	27.3	
3	353	49.0	542	43.9	
4	79	11.0	125	10.1	
Dichotomized groups					0.012
0-2	289	40.1	567	46.0	
3-4	432	59.9	667	54.0	

^aThe number represents the numbers of risk genotypes; the risk genotypes used for the calculation were 16195CC, 16286CC, 45996GT+TT, and 46346CA+AA genotypes).

^bTwo-sided chi-square test for the combined genotype distributions between the cases and controls.

Table 5
Association and Stratification analyses between the combined genotypes of *MGMT* polymorphisms and SCCHN risk

Variables	n (case/control)	Combined genotypes (case/control) ^a				Crude OR (95% CI)	Adjusted OR (95% CI) ^b
		0-2 risk genotypes		3-4 risk genotypes			
		n	%	n	%		
All subjects	721/1,234	289/567	40.1/46.0	432/667	59.9/54.0	1.27 (1.06-1.53)	1.27 (1.05-1.53)
Age (years)							
≤ 45	105/195	40/104	38.1/53.3	65/91	61.9/46.7	1.86 (1.14-3.01)	1.81 (1.11-2.96)
46-60	334/532	134/233	40.1/43.8	200/299	59.9/56.2	1.21 (0.90-1.62)	1.21 (0.91-1.61)
> 60	282/507	115/230	40.8/45.4	167/277	59.2/54.6	1.16 (0.88-1.54)	1.16 (0.86-1.58)
Sex							
Female	181/320	72/152	39.8/47.5	109/168	60.2/52.5	1.37 (0.95-1.98)	1.33 (0.91-1.94)
Male	540/914	217/415	40.2/45.4	323/499	59.8/54.6	1.24 (1.00-1.54)	1.24 (1.00-1.55)
Smoking status							
Never	188/371	73/168	38.8/45.3	115/203	61.2/54.7	1.30 (0.91-1.86)	1.30 (0.91-1.87)
Ever	533/863	216/399	40.5/46.2	317/464	59.5/53.8	1.26 (1.01-1.57)	1.25 (1.01-1.56)
Drinking status							
Never	158/374	65/173	41.1/46.3	93/201	58.9/53.7	1.23 (0.84-1.79)	1.24 (0.85-1.81)
Ever	563/860	224/394	39.8/45.8	339/466	60.2/54.2	1.28 (1.03-1.59)	1.29 (1.04-1.60)
Tumor site							
Oral cavity	222/1,234	93/567	41.9/46.0	129/667	58.1/54.0	1.18 (0.88-1.58)	1.18 (0.88-1.58)
Oropharynx	326/1,234	121/567	37.1/46.0	205/667	62.9/54.0	1.44 (1.12-1.85)	1.45 (1.12-1.87)
Larynx ^c	173/1,234	75/567	43.4/46.0	98/667	56.6/54.0	1.11 (0.81-1.53)	1.10 (0.80-1.53)

^aThe number represents the numbers of risk genotypes; the risk genotypes used for the calculation were 16195CC, 16286CC, 45996GT+TT, and 46346CA+AA genotypes).

^bORs were adjusted for age, sex, smoking status, and alcohol use in a logistic regression model.

^cIncluded both hypopharynx and larynx.

Table 6

Associations of the combined genotypes of *MGMT* polymorphisms with regional lymph nodes and tumor stage of oropharyngeal cancer^a

Combined genotypes (No. of risk genotypes) ^b	No regional lymph node metastasis (N ₀)		Involvement of regional lymph nodes (N ₁₋₃)	
	Case/control (%)	Adjusted OR (95% CI) ^c	Case/control (%)	Adjusted OR (95% CI) ^c
0-2	21/567 (43.7/46.0)	1.00	100/567 (36.0/46.0)	1.00
3-4	27/667 (56.3/54.0)	1.10 (0.61-1.97)	178/667 (64.0/54.0)	1.52 (1.16-1.89)

Combined genotypes (No. of risk genotypes) ^b	T ₁₋₂		T ₃₋₄	
	Case/control (%)	Adjusted OR (95% CI) ^c	Case/control (%)	Adjusted OR (95% CI) ^c
0-2	65/567 (33.0/46.0)	1.00	56/567 (43.4/46.0)	1.00
3-4	132/667 (67.0/54.0)	1.77 (1.28-2.44)	73/667 (56.6/54.0)	1.09 (0.76-1.58)

^aN, regional lymph node involvement. N₀, no regional lymph nodes; N₁, N₂, and N₃, increasing involvement of regional lymph nodes [40].

^bThe number represents the numbers of risk genotypes; the risk genotypes used for the calculation were 16195CC, 16286CC, 45996GT+TT, and 46346CA+AA genotypes).

^cORs were obtained from a multivariate logistic regression model with adjustment for age, sex, smoking status, and alcohol use.

Table 7
False positive reporting probability values for Associations between the combined genotypes of *MGMT* and *SCCHN* risk

Combined Genotypes	Positive OR and 95% CI ^a	<i>p</i> ^b	Statistical Power ^c	Prior probability				
				0.25	0.1	0.01	0.001	0.0001
0-2 risk genotypes vs. 3-4 risk genotypes								
All subjects	1.27 (1.06-1.53)	0.012	0.958	0.036	0.101	0.553	0.926	0.992
Ever smoker	1.26 (1.01-1.57)	0.037	0.937	0.106	0.262	0.796	0.975	0.997
Ever drinking	1.28 (1.03-1.59)	0.025	0.923	0.075	0.196	0.728	0.964	0.996
Oropharynx	1.44 (1.12-1.85)	0.004	0.615	0.019	0.055	0.392	0.867	0.985
Oropharyngeal Cancer								
Involvement of regional lymph nodes(N ₁₋₃)	1.51 (1.16-1.98)	0.003	0.499	0.018	0.051	0.373	0.857	0.984
Tumor stage T ₁₋₂	1.73 (1.26-2.37)	0.001	0.227	0.013	0.038	0.304	0.815	0.978

^aThe crude OR.

^bThe omnibus chi-square test of the combination genotype distributions.

^cCalculated using study subjects to detect an OR of 1.5 with the common combined genotype used as the reference.